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Control of Synaptic Strength, a Novel Function of Akt

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Summary

Akt (also known as PKB), a serine/threonine kinase involved in diverse signal-transduction pathways, is highly expressed in the brain. Akt is known to have a strong antiapoptotic action and thereby to be critically involved in neuronal survival, but its potential role in the dynamic modulation of synaptic transmission is unknown. Here we report that Akt phosphorylates, both in vitro and in vivo, the type A γ-aminobutyric acid receptor ($GABA_AR$), the principal receptor mediating fast inhibitory synaptic transmission in the mammalian brain. Akt-mediated phosphorylation increases the number of $GABA_A$Rs on the plasma membrane surface, thereby increasing the receptor-mediated synaptic transmission in neurons. These results identify the $GABA_AR$ as a novel substrate of Akt, thereby linking Akt to the regulation of synaptic strength. This work also provides evidence for the rapid regulation of neurotransmitter receptor numbers in the postsynaptic domain by direct receptor phosphorylation as an important means of producing synaptic plasticity.

Introduction

Akt/PKB, a serine/threonine kinase, is the cellular homolog of the retroviral oncogene v-Akt and shares high homology with protein kinase A (PKA) and protein kinase C (PKC). Three identified isoforms, α, β, and γ, all contain an N-terminal pleckstrin homology (PH) domain, a central kinase domain, and a C-terminal regulatory domain (Coffer et al., 1998; Datta et al., 1999; Downward, 1998). Akt is activated rapidly by a variety of extracellular factors including insulin and growth factors and intracellular signaling molecules such as Ras (Bhave et al., 1999; Downward, 1998; Dudek et al., 1997; Yano et al., 1998). Upon activation, Akt phosphorylates a variety of substrate proteins at the serine/threonine residue of a consensus sequence, RXRXXS/T (reviewed in Datta et al., 1999), thereby inducing diverse biological responses, such as inhibiting programmed cell death (apoptosis), promoting cell proliferation, and initiating a number of metabolic responses (Datta et al., 1999; Downward, 1998). Akt is not only expressed in a variety of peripheral tissues, but is also highly expressed in the central nervous system (CNS). In the CNS, activation of Akt by growth factor stimulation has recently been shown to play an important role in regulating neuronal cell survival (Datta et al., 1999; Dudek et al., 1997). But, whether it, like many kinases expressed in the CNS, is involved in the mediation of neuronal functioning by regulating synaptic transmission and plasticity has not previously been addressed.

The A type γ-aminobutyric acid receptor ($GABA_AR$) is a ligand-gated Cl ion channel that mediates fast synaptic transmission at the vast majority of inhibitory synapses in the mammalian brain and is also the site of action of many psychoactive drugs, such as benzodiazepines and barbiturates (Macdonald and Olsen, 1994; McKernan and Whiting, 1996). $GABA_A$Rs are thought to be heteropentameric structures, assembled by combining homologous subunits from various subfamilies including α (1-6), β (1-3), γ (1-3), δ, ϵ, and θ (Macdonald and Olsen, 1994; McKernan and Whiting, 1996). Each of the subunits contains four α-helical hydrophobic transmembrane domains (Figure 1A). The subunit composition of $GABA_A$Rs is important to receptor expression and membrane trafficking, thereby affecting its functional integrity and pharmacological properties. Coexpression of an α and a β subunit is the minimum requirement for surface expression of functional $GABA_A$Rs in a heterologous cell line, and the full pharmacological profile of native $GABA_A$Rs requires the α, β, and γ subunits. The most abundant population of native $GABA_A$Rs in the mammalian brain is believed to be the $\alpha_1\beta_2\gamma_2$ subunit combination (McKernan and Whiting, 1996). The functional modification of the $GABA_AR$ is fundamental for brain function and dysfunction, and such modifications have mainly been thought to involve altering the properties of the existing receptor channels, such as channel gating and conductance (Eghbali et al., 1997; Macdonald and Twyman, 1992). However, a rapid change in receptor number at the postsynaptic domain has recently been suggested to be an efficient means of regulating receptor function, and hence receptor-mediated synaptic transmission (Nusser et al., 1998; Wan et al., 1997b). We have recently demonstrated that stimulation of hippocampal neurons with insulin results in an increase in the number of functional postsynaptic $GABA_A$Rs by a rapid recruitment of the receptors from the

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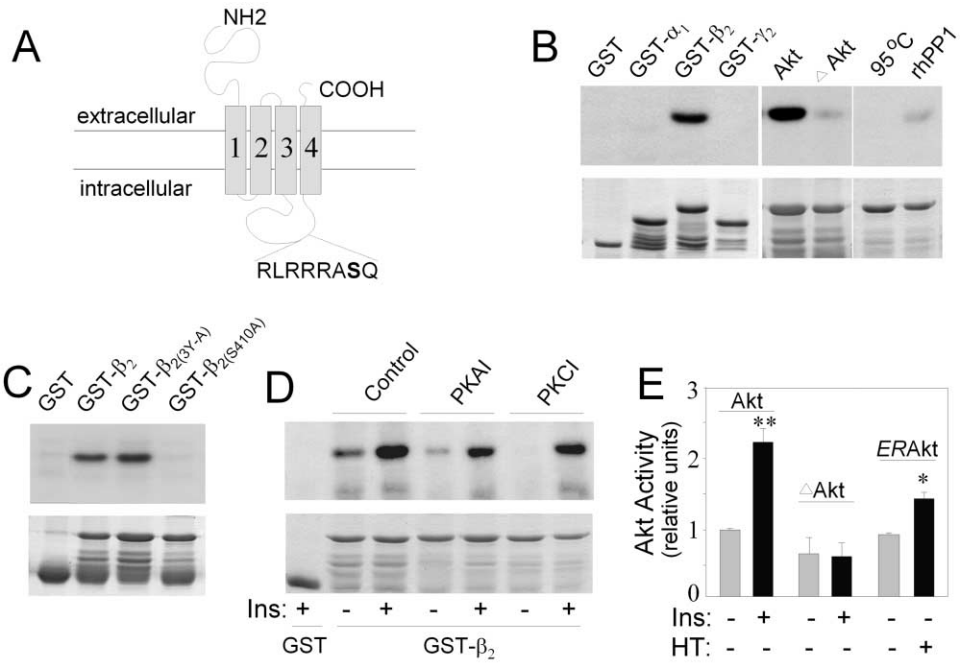

Figure 1. In Vitro Phosphorylation of S410 of the $GABA_A$ Receptor β_2 Subunit by Akt

(A) Proposed schematic representation of $GABA_AR$ subunit plasma membrane topology.

(B) Left: GST fusion proteins containing the intracellular loop between transmembrane domains 3 and 4 of α_1 (GST-α_1), β_2 (GST-β_2), or γ_2 (GST-γ_2) $GABA_AR$ subunits, along with GST alone as a control, were phosphorylated by immunopurified active Akt (Akt) in an in vitro phosphorylation assay, and then subjected to SDS-PAGE and analysis by autoradiography (top). Coomassie blue staining of the same SDS-PAGE gel shows that a similar amount of GST fusion protein was used in each reaction (bottom). Right: GST-β_2 fusion proteins were incubated with active Akt (Akt), kinase-dead Akt (ΔAkt), or heat-inactivated Akt (95°C). In the last lane, GST-β_2 was first phosphorylated and then treated with the specific serine/threonine phosphatase, recombinant human protein phosphatase 1 (rhPP1).

(C) Site-directed mutagenesis of GST-β_2 converting S410 into an alanine (GST-$\beta_{2(S410A)}$) eliminated phosphorylation of the GST fusion protein by active Akt, while in contrast, Akt-induced phosphorylation was not affected by mutating all three tyrosine residues present within the GST-β_2 loop (GST-$\beta_{2(3Y-A)}$).

(D) GST-β_2 was incubated, in the absence (Control) or presence of the PKA inhibitor Rp-cAMP (PKAI; 50 μM) or the PKC inhibitor Gö6850 (PKCI; 125 nM), with Akt immunopurified from HEK293 cells transiently expressing HA-Akt that were treated with (+) or without (−) insulin (200 nM, 5 min).

(E) HEK293 cells were transiently transfected with HA-epitope-tagged wild-type Akt (Akt), kinase-dead Akt (ΔAkt), or estrogen receptor (ER) agonist hydroxyltamoxifen (HT)-dependent, conditionally active Akt (ERAkt). Cells were treated with (+) or without (−) insulin (Ins; 200 nM, 5 min) or HT (1 μM, 30 min). The various forms of Akt were immunoprecipitated with an anti-HA antibody and their kinase activity was determined in an immunocomplex assay using the specific Akt substrate, Crosstide. Paired t tests were used to analyze differences between control and treated groups: * and ** denote $p < 0.05$ and $p < 0.01$, respectively (mean ± SE; $n = 3$ in each group). Note that Akt and ΔAkt immunopurified from insulin-stimulated cells were used in the reactions shown in (B)–(D), and Akt from either insulin-treated or -untreated cells was used in reactions shown in (E).

intracellular compartment to postsynaptic domains in a β_2 subunit-dependent manner (Wan et al., 1997b). Although the detailed mechanisms underlying insulin-induced receptor translocation remain unknown, previous studies have demonstrated that β subunits are critical determinants for plasma membrane expression of heteromeric $GABA_ARs$ (Connolly et al., 1996; Perez-Velazquez and Angelides, 1993; Wan et al., 1997a). Interestingly, by examining the sequences of members of the major $GABA_AR$ subunit families, we found that a short, serine-containing sequence (RLRRRA**S**Q; Figure 1A), very similar to the consensus sequence of Akt kinase, is present in the major intracellular loop between the third and fourth transmembrane domains of all β subunits. As Akt plays an important role in mediating insulin action on GLUT4 translocation and glucose transport in peripheral insulin-responsive tissues (Datta et al., 1999; Downward, 1998), we set out to determine whether Akt phosphorylates $GABA_AR$ β subunits and regulates cell-surface expression of the receptor, and hence is involved in control of the strength of $GABA_AR$-mediated synaptic transmission.

Results

Akt Phosphorylates the $GABA_AR$ β_2 Subunit at Serine 410 In Vitro and In Vivo

To determine whether the major intracellular loop of β subunits of $GABA_ARs$ is a substrate for Akt, we first performed in vitro phosphorylation assays to examine the ability of purified active Akt kinase to phosphorylate glutathione S-transferase (GST) fusion proteins containing the intracellular loop between the third and fourth transmembrane domains of $GABA_AR$ β_2 (GST-β_2). GST-α_1 and GST-γ_2 intracellular loops that do not contain the potential Akt phosphorylation site, along with the GST

backbone, were used as controls (Figure 1). Wild-type Akt and a functionally inactive mutant form (ΔAkt; Akt_{K179A}) were immunopurified from insulin-stimulated (200 nM, 5 min) HEK293 cells transiently transfected with the corresponding cDNAs. The kinase activity was then assayed in an in vitro immunocomplex kinase assay using a specific Akt substrate (Figure 1E). We found that active Akt was able to phosphorylate GST-β_2, but not GST alone, GST-α_1, or GST-γ_2, in vitro (Figure 1B). In contrast, GST-β_2 was not phosphorylated by the kinase-dead mutant, ΔAkt, or heat-inactivated wild-type Akt, indicating the requirement for Akt kinase activity in this phosphorylation (Figure 1B). Consistent with phosphorylation at a serine/threonine site(s), phosphorylation of GST-β_2 by Akt could be reversed by the recombinant human protein phosphatase 1 (rhPP1), a serine/threonine-specific phosphatase (Figure 1B). We next examined whether this phosphorylation occurred at serine 410 (S410), within the predicted short amino acid stretch of the β_2 loop, using site-directed mutagenesis. As shown in Figure 1C, the point mutation that converts S410 into an alanine (GST-$\beta_{2(S410A)}$) completely eliminated the Akt-mediated phosphorylation of GST-β_2, while as a control, mutation of the three tyrosine residues present in the same intracellular loop (GST-$\beta_{2(3Y\text{-}A)}$) failed to alter Akt-induced phosphorylation levels. Thus, S410 is the only residue within the intracellular loop of the β_2 subunit that can be phosphorylated by Akt in vitro. The same site has also been proposed to be phosphorylated by both PKA and PKC in vitro (McDonald and Moss, 1997). However, the phosphorylation of GST-β_2 persisted in the presence of inhibitors for PKA and PKC (Figure 1D) at concentrations that were sufficient to block the in vitro phosphorylation of the same substrate by purified PKA or PKC (data not shown). Taken together, these results demonstrate that the major intracellular loop of the $GABA_AR$ β_2 subunit is a substrate of Akt in vitro, and that phosphorylation occurs at a single specific site, S410.

To determine whether $GABA_ARs$ are substrates of Akt kinase in vivo, HEK293 cells were transiently transfected with cDNAs encoding $GABA_AR$ α_1^{Flag}, β_2, and γ_2 subunits and either wild-type Akt or kinase-dead ΔAkt. These cells were labeled with [^{32}P]orthophosphate 48 hr after transfection. Following activation of Akt with insulin (200 nM, 5 min) (Dudek et al., 1997), heteromeric $GABA_ARs$ were immunoprecipitated with anti-Flag antibody under nondenaturing conditions and resolved by SDS-PAGE. As shown in Figure 2A, autoradiographic analysis revealed a specific, although faint, phosphorylated band in $GABA_AR$- and wild-type Akt-transfected, but not in mock transfected, cells. As this phosphorylated band migrates at the predicted molecular weight for the $GABA_AR$ β_2 subunit (58 kDa), the result is in good agreement with the in vitro phosphorylation results (Figure 1B), suggesting that the β_2, but not the α_1 or γ_2, subunit is the major substrate for basal in vivo phosphorylation. Direct immunoprecipitation of the phosphorylated β_2 subunit using anti-myc antibody from cells transiently transfected with $GABA_AR$ α_1, β_2^{myc}, and γ_2 subunits and Akt (Figure 2B) under denaturing conditions further confirmed this result. Insulin stimulation dramatically increased the level of β_2^{myc} subunit phosphorylation in cells coexpressing Akt. Since this increase in β_2 subunit phosphorylation was largely attenuated by coexpressing ΔAkt (Figure 2A) but was not affected by inhibitors for PKA or PKC (Figure 2B), Akt kinase activity appears to be required for the insulin-induced enhancement of β_2 phosphorylation. In order to determine whether activation of Akt was sufficient to induce β_2 phosphorylation, we next cotransfected HEK293 cells with $GABA_AR$ subunits and a conditionally active form of Akt (ER-Akt) (Kohn et al., 1998). ER-Akt is a chimeric protein containing a constitutively active Akt and the hormone binding domain of a mutant murine estrogen receptor (ER) that specifically recognizes 4-hydroxytamoxifen (HT). ER-Akt is inactive until the ER region selectively binds its agonist, HT (Figure 1E). As expected, activation of ER-Akt by HT, which bypasses the insulin-stimulated signaling pathway, significantly increased the level of β_2 phosphorylation, demonstrating that Akt kinase activation is sufficient for in vivo phosphorylation of the β_2 subunit (Figure 2A).

To determine whether S410 of the β_2 subunit is the in vivo substrate of Akt, a β_2 mutant ($\beta_{2(S410A)}$) was constructed in which S410 was mutated into an alanine using site-directed mutagenesis. As shown in Figure 2C, the single S410A mutation dramatically reduced the level of insulin-induced phosphorylation of the β_2 subunit. These results are consistent with the in vitro phosphorylation assay (Figure 1C) and indicate that S410 is the sole site in the β_2 subunit that is phosphorylated by Akt in vivo.

Activation of Akt Increases Cell Surface Expression of Recombinant $GABA_ARs$

Insulin stimulation causes rapid and β subunit-dependent translocation of intracellular $GABA_ARs$ to the plasma membrane surface, thereby potentiating receptor-mediated current responses (Wan et al., 1997b). To test a potential role of phosphorylation of the β_2 subunit by Akt in this receptor translocation, we next examined the subcellular localization of the $GABA_ARs$ in HEK293 cells expressing α_1^{Flag}, β_2, and γ_2 subunits. $GABA_ARs$ expressed on the plasma membrane surface and in the intracellular compartment(s) were detected by confocal immunofluorescent microscopy and quantitated using colorimetric cell-ELISA assays following labeling of the Flag epitope of α_1^{Flag} subunits under nonpermeabilized and permeabilized conditions, respectively (Figure 3). Transfected $GABA_ARs$ were found to be expressed both on the plasma membrane surface and in the intracellular compartment under basal conditions. Insulin stimulation (200 nM, 10 min) resulted in a rapid translocation of receptors from the intracellular compartment to the plasma membrane and hence increased the cell-surface levels of $GABA_ARs$ (Figures 3A, left panel, and 3B), confirming our previous results (Man et al., 2000; Wan et al., 1997b). Prior treatment of these cells with inhibitors for PKA or PKC had no detectable effect on the insulin action, excluding the participation of these kinases in the insulin-induced receptor translocation (Figure 3B). However, cotransfection of the kinase-dead ΔAkt, while having little effect on the basal level of cell-surface expression, blocked the insulin-induced increase of cell surface $GABA_ARs$. As controls, overexpression of wild-type (wt)-Akt (and mock vectors, data not shown) did

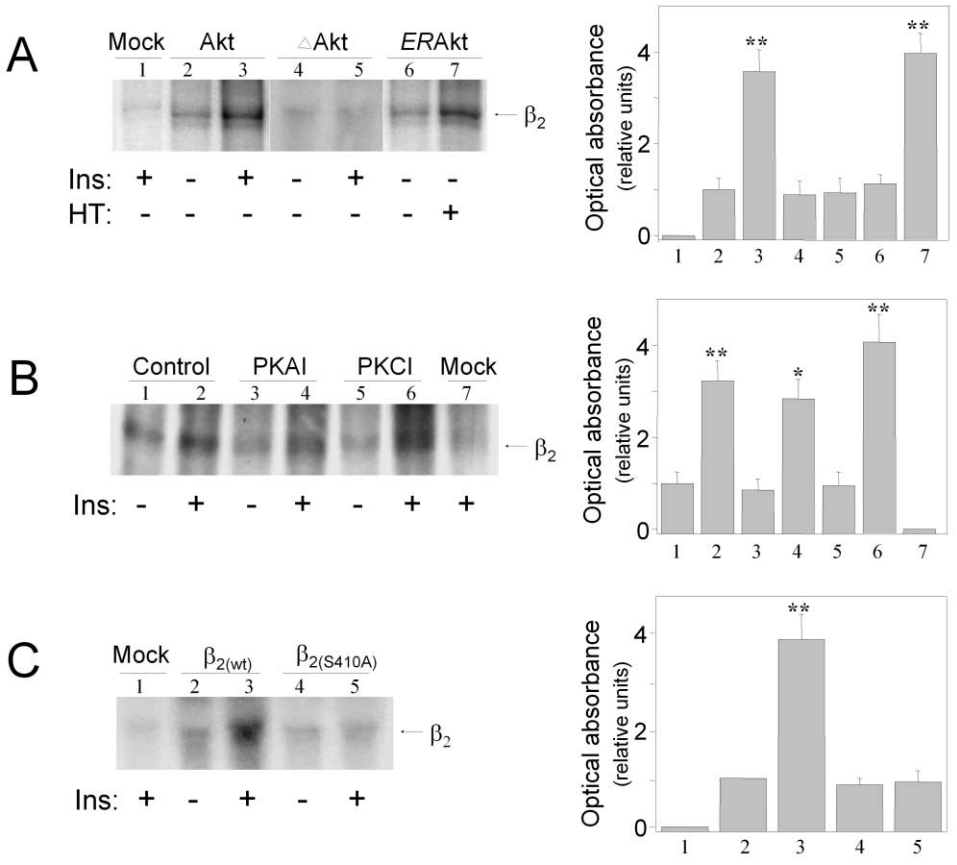

Figure 2. Specific Phosphorylation of $GABA_A$ Receptor β_2 Subunit by Akt In Vivo

(A and B) HEK293 cells transiently expressing $GABA_AR$ α_1^{Flag}, β_2, and γ_2 subunits and either wild-type Akt (Akt), kinase-dead Akt (ΔAkt), or the estrogen receptor (ER) agonist hydroxyltamoxifen (HT)-dependent, conditionally active Akt (ERAkt) were serum deprived and labeled with [^{32}P]orthophosphate for a minimum of 3 hr. Cells were treated, in (A), with (+) or without (−) insulin (Ins; 200 nM, 5 min) or HT (1 μM, 30 min), or treated, in (B), with or without insulin in the absence or presence of the PKA inhibitor Rp-cAMP (PKAI; 50 μM, 15 min) or the PKC inhibitor Gö6850 (PKCI; 125 nM, 15 min). $GABA_AR$ complexes were immunoprecipitated with an anti-Flag antibody under nondenaturing conditions and subjected to SDS-PAGE and autoradiography. The major phosphorylated band migrates at 58 kDa, the predicted molecular weight of the $GABA_AR$ β_2 subunit.

(C) HEK293 cells were transiently cotransfected with either myc epitope-tagged wild-type β_2^{myc} or the mutant $\beta_{2(S410A)}^{myc}$, along with α_1, γ_2, and Akt. Following [^{32}P]orthophosphate labeling and insulin treatment as described above, the $GABA_AR$ β_2 subunit was immunoprecipitated with anti-myc antibody under denaturing conditions and subjected to SDS-PAGE and autoradiography. Densitometric quantitation from 3–4 separate experiments is summarized in the histograms in the right-hand panels (*$p < 0.05$, **$p < 0.01$).

not affect either the basal or insulin-induced increase in cell surface $GABA_ARs$. In addition, activation of phosphatidylinositol 3-kinase (PI3K) is known to be an essential step in the activation of Akt by a number of growth factors, including insulin. We found that cotransfection of the dominant-negative interfering PI3K (ΔPI3K) abolished the insulin-stimulated increase of cell surface $GABA_ARs$ (Figure 3B), further supporting the hypothesis that activation of Akt is an absolute necessity for insulin-induced $GABA_AR$ translocation. Furthermore, in cells coexpressing ER-Akt, HT treatment mimicked insulin's actions, resulting in an increase in the $GABA_AR$ cell surface expression (Figure 3B). These results suggest that activation of Akt is sufficient to lead to $GABA_AR$ translocation. Consistent with the critical role of the direct phosphorylation of β_2 S410 in Akt-dependent receptor translocation, the single S410A site mutation ($\beta_{2(S410A)}$) completely abolished the insulin-induced $GABA_AR$ translocation (Figures 3A, right panel, and 3B).

Activation of Akt Increases the Number of Postsynaptic $GABA_ARs$ and Receptor-Mediated Synaptic Transmission in Neurons

Since Akt is highly expressed in neurons and can be activated following stimulation of neurons by a number of neuronal growth factors and transmitters, Akt-mediated rapid increase in $GABA_ARs$ may have an important role in the regulation of receptor-mediated synaptic transmission. This prediction was investigated in cultured rat hippocampal neurons. We first ascertained whether Akt in neurons, particularly at the GABAergic synapses, could be rapidly activated following stimulation by growth factors such as insulin. Phosphorylation of Thr308 is a prerequisite for Akt activation, and phosphorylation of Ser473 is also required for full activation of the kinase; hence, the determination of the phosphorylation levels of Thr308 and/or Ser473 has been used as an indicator of Akt activation in a variety of tissues in situ (Coffer et al., 1998). We therefore examined insulin

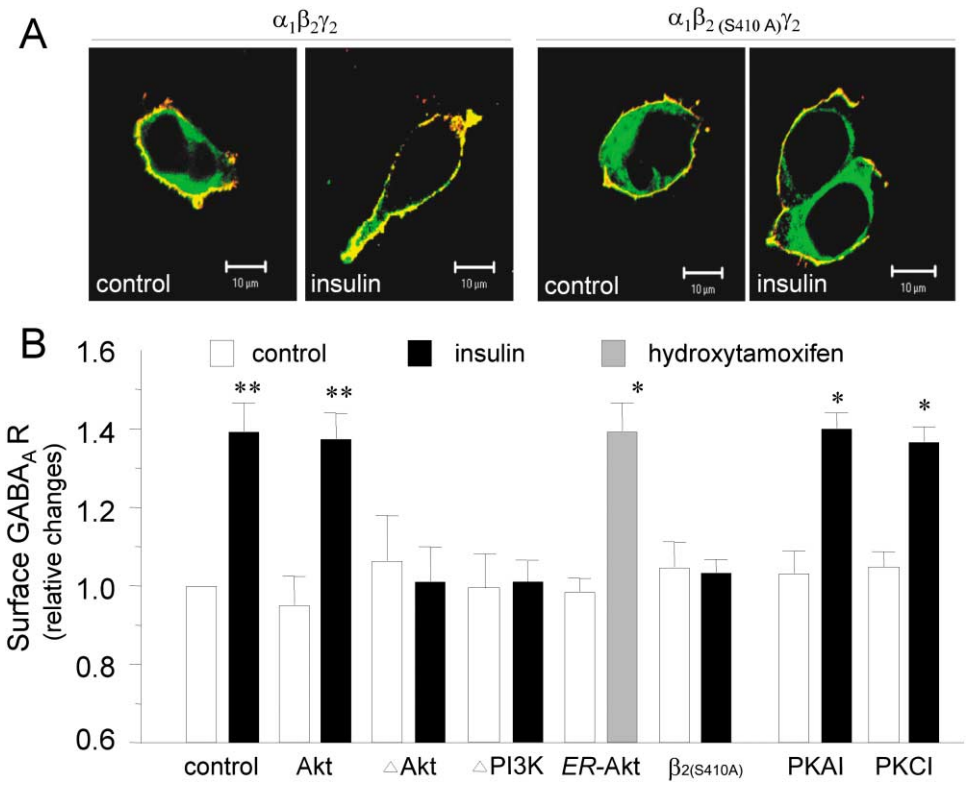

Figure 3. Translocation of $GABA_A$ Receptors to the Plasma Membrane Requires Akt-Mediated Phosphorylation of β_2 S410

Plasmid transfections and various treatments of HEK293 cells were the same as described in Figure 2.

(A) Superimposed confocal sections of immunofluorescent staining of cell surface (red) and intracellular (green) $GABA_ARs$ with an antibody against the N-terminal Flag epitope-tagged extracellular domain of the α_1 subunit. The effects of insulin stimulation (200 nM, 10 min) were compared in cells transfected with wild-type β_2 subunit (left) and the S410A mutant form ($\beta_{2(S410A)}$; right).

(B) Cell surface $GABA_AR$ expression was quantitively analyzed by a cell-ELISA assay, using the ratio of absorbance readings obtained with anti-Flag antibody labeling under nonpermeabilized (surface) versus permeabilized (total) conditions. Changes in the cell surface expression of the receptor [Surface $GABA_AR$ (relative changes)] were then determined by normalizing the ratio of cell surface/total receptors for individual Akt transfections and/or drug treatments against that obtained for non-Akt-transfected and nontreated control conditions (Control). Paired t tests were used to analyze differences between control and drug-treated groups: * and ** denote $p < 0.05$ and $p < 0.01$, respectively (mean ± SE; $n = 6$ in each group).

activation of Akt in cultured hippocampal neurons using an antibody raised specifically against phosphorylated Akt-Thr308. As shown in Figure 4B, under basal conditions there was a relatively low level of phosphorylated endogenous Akt, which was mainly concentrated within the soma and large proximal dendrites. Double staining of the neurons with an antibody against the $GABA_AR$ β_2 subunit indicated that the phosphorylated Akt was not colocalized with $GABA_ARs$ (Figure 4A and insets a and b). Insulin stimulation (200 nM, 10 min) caused a dramatic increase in the amount of phosphorylated Akt. Most interestingly, the phosphorylated Akt was exclusively present in remote dendrites and formed numerous small puncta that colocalized with $GABA_AR$ clusters (Figure 4A and insets c and d). These results suggest that, following insulin stimulation, there may be a localized activation of PI3K and a consequent accumulation of PI3K lipid products, PtdIns-3P, within dendritic synapses. The increased concentration of PtdIns-3P in these synapses would in turn induce a recruitment and activation of Akt to the vicinity of $GABA_ARs$.

To confirm these predictions, we transfected cultured hippocampal neurons with cDNA plasmids encoding green fluorescent protein (GFP), or chimeric proteins of GFP and Akt (GFP-Akt) or the AH domain of Akt (GFP-AH) (Figure 4B, d). GFP-AH contains the Akt binding domain for PtdIns-3P (also known as the pleckstrin homology (PH) domain) that is required for the translocation of Akt to the plasma membrane and hence its activation by plasma membrane-associated lipid kinases. Plasma membrane recruitment of GFP-Akt or GFP-AH has previously been used to show in situ PI3K activation in live cells (Watton and Downward, 1999). The dynamic movement of these GFP constructs was then monitored in the same neurons before and during insulin stimulation using real-time confocal microscopic techniques. All of GFP, GFP-Akt, and GFP-AH had similar expression patterns in the transfected neurons, being found in both cell soma and dendrites (Figure 4B, a). Their distribution patterns were relatively stable and no detectable spontaneous movement was evident over 30 min of constant monitoring under basal conditions. However, as expected, brief insulin stimulation (200 nM, 10 min), while having no obvious effect on GFP movement (data not shown), resulted in a rapid increase in the dynamics of GFP-Akt and GFP-AH (Figure 4B, b). Although dynamic

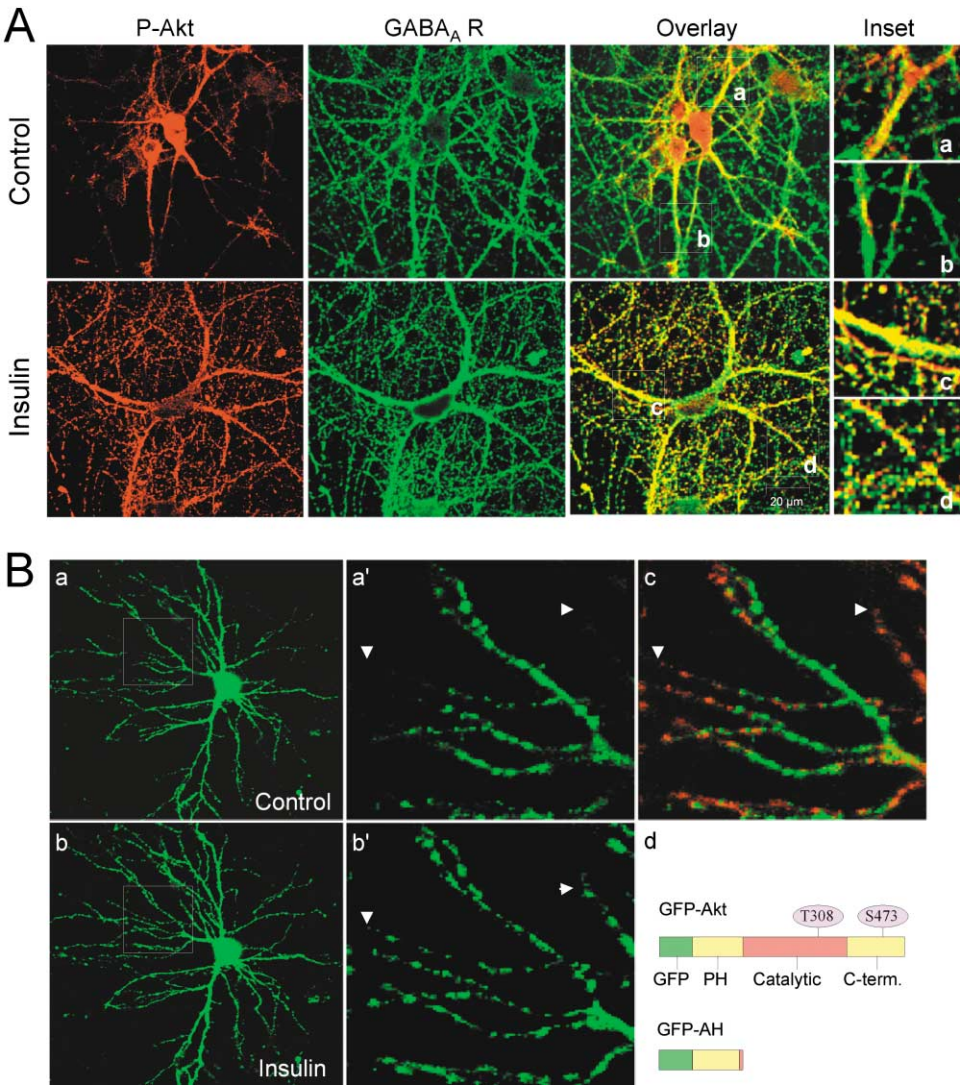

Figure 4. Insulin Translocates and Activates Akt in Cultured Hippocampal Neurons

(A) Cultured hippocampal neurons were treated without (Control) or with insulin (Insulin; 200 nM, 10 min) and stained for activated Akt using an anti-Akt-phosphoThr308 antibody (P-Akt; red). The GABA$_A$R was sequentially stained using an antibody against the β_2 subunit (green). Insets from the overlay images are shown enlarged in the right panels.

(B) Cultured hippocampal neurons were transfected with a GFP-AH construct that encodes GFP fused with a large portion of the PH domain of Akt, as illustrated in d. The dynamic movement of GFP-AH was monitored using real-time confocal microscopic techniques. a and b are images obtained before (Control) and 10 min after insulin treatment (Insulin; 200 nM), respectively. a′ and b′ are enlargements of the indicated areas in a and b, respectively. To obtain c, the image a′ was subtracted from b′ to obtain the difference of the GFP-Akt redistribution produced by insulin, and the resulting image (red) was then overlaid with the control image a′ (green). Insulin produced an overall redistribution of GFP-AH from soma and proximal dendrites to remote dendritic terminals (white arrowheads indicate examples where obvious translocations can be readily observed). d shows a schematic illustration of GFP-tagged wild-type Akt (GFP-Akt) and the mutated form of Akt (GFP-AH). The location of phosphorylation sites required for full activation of Akt kinase activity are indicated.

movement of GFP-Akt could be observed in both directions, i.e., from the cell soma to remote dendrites and vice versa, there was a clear overall redistribution of the clusters of GFP-tagged Akt from the soma and proximal dendrites to the remote dendrites (Figure 4B, a′ and b′), and this is evident in the red pseudo-colored image shown in Figure 4B, inset c, which was obtained by subtracting the image of GFP-AH in a hippocampal neuron under basal nonstimulated conditions (Figure 4B, a′) from that obtained in the same neuron 10 min after insulin stimulation (Figure 4B, b′). These results correspond well with phospho-Thr308 staining in Figure 4A, strongly suggesting that, following insulin stimulation, there was a recruitment and activation of Akt within the GABAergic synapses, and by this means Akt is put in a strategic position to exert its role in phosphorylating and translocating GABA$_A$Rs, thereby modulating receptor-mediated synaptic transmission.

We next examined the phosphorylation of native GABA$_A$R β_2 subunits at S410 in cultured neurons in situ using an antibody against the Akt phosphomotif (RXRXXpS/T), also referred to as phospho Akt substrate

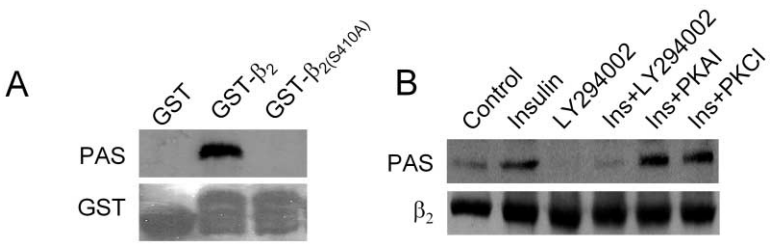

Figure 5. In Situ Phosphorylation of $GABA_A$ Receptor β_2 S410 by Akt in Neurons

(A) GST-β_2 and GST-$\beta_{2(S410A)}$, along with GST alone as a control, were exposed to active recombinant Akt in an in vitro phosphorylation assay and then subjected to SDS-PAGE. The membrane was sequentially probed with anti-PAS (top blot; PAS) and anti-GST antibodies (bottom blot; GST).

(B) Control neuronal cultures (Control) and cultures treated with insulin (200 nM; 5 min) in the absence (Insulin) or presence of LY294002 (+ LY294002; 10 μM), the PKA inhibitor Rp-cAMP (+PKAI; 50 μM), or the PKC inhibitor Gö6850 (+PKCI; 125 nM). Native β_2 subunits were isolated by immunoprecipitation of culture lysates with a monoclonal anti-β_2 antibody under denaturing conditions, and phosphorylation of β_2 S410 was detected by Western blotting with anti-PAS antibody (top blot; PAS). The efficiency of β_2 immunoprecipitations was determined by reprobing the same membrane with a polyclonal anti-β_2 antibody (bottom blot; β_2).

(PAS) (Kane et al., 2002). An in vitro phosphorylation assay determined that the antibody specifically recognized Akt-phosphorylated GST-β_2, but not GST alone (Figure 5A). Furthermore, mutation of GST-β_2 S410 to alanine (GST-$\beta_{2(S410A)}$) eliminated immunoreactivity with the anti-PAS antibody. To examine the insulin-induced Akt phosphorylation of native β_2 S410, cultured neurons with or without insulin treatment (200 nM, 5 min) were subjected to immunoprecipitation of native $GABA_AR$ β_2 subunits. The immunoprecipitates, when immunoblotted with anti-PAS antibody, demonstrated a low level of phosphorylation of β_2 S410 under basal conditions, and the level increased following insulin stimulation (Figure 5B; PAS). Both basal and insulin-stimulated phosphorylation seemed to be mediated by Akt activity, as they were both inhibited by LY294002, a specific inhibitor of PI3K, which is a necessary signaling molecule upstream of Akt (Downward, 1998). In contrast, both PKA and PKC inhibitors failed to reduce insulin-induced phosphorylation of β_2 S410 (Figure 5B). These results suggest endogenous Akt is the primary kinase involved in insulin-stimulated phosphorylation of native β_2 S410 in the cultured hippocampal neurons used in this study.

We then investigated the potential role of Akt phosphorylation in insulin-induced recruitment of postsynaptic functional $GABA_ARs$, and hence potentiation of the receptor-mediated inhibitory postsynaptic currents (IPSCs) in these neurons (Wan et al., 1997b). Due to the lack of a specific Akt inhibitor, we employed GFP-AH as a dominant-negative Akt (Figure 4B, d; Watton and Downward, 1999). As predicted, the purified GFP-AH, unlike its wild-type counterpart, lacked detectable Akt kinase activity, failing to phosphorylate GST-β_2 in vitro (Figure 6A), and when expressed in HEK293 cells, it inhibited wild-type Akt kinase activity in a dominant-negative manner (Figure 6B). Transiently transfected hippocampal neurons routinely resulted in a 2%–3% rate of expression. Such low transfection efficiency was a great advantage in the present study, as it allowed us to select a single transfected neuron that was surrounded and innervated by multiple nontransfected neurons for use in our recordings (Figure 6C). This ensured the dominant-negative effect of the GFP-AH was limited to postsynaptic neurons undergoing whole-cell recording. As previously reported (Wan et al., 1997b), brief application of insulin (0.5 μM, 10 min) in control, nontransfected neurons increased both mIPSC amplitude and frequency, without altering their time courses (Figure 6F). Transfection of neurons with either GFP-Akt or GFP alone did not significantly alter insulin's ability to potentiate the mIPSCs. In contrast, in cells overexpressing GFP-AH (Figures 6E and 6F), insulin failed to alter either the mIPSC amplitude or frequency. The dominant-negative effect of GFP-AH was mimicked by the overexpression of Akt-AAA (Figure 6F), another dominant-negative Akt mutant that carries three mutations: K179A, resulting in loss of kinase activity, and T308A and S473A, which result in loss of ability to be phosphorylated and activated by PDK1 and other kinases (Coffer et al., 1998). However, the insulin potentiation of mIPSCs was not affected by inhibition of either PKA or PKC activity (Figure 6F). The results indicate that endogenous Akt, but not PKA or PKC, is necessary and sufficient to mediate insulin-induced potentiation of mIPSCs.

The changes in mIPSC amplitude without alterations in time course are consistent with a rapid increase in the number of functional $GABA_ARs$ at the postsynaptic domain following activation of Akt, and this is also in full agreement with the results shown in Figure 3. In order to provide direct evidence that activation of Akt leads to the rapid cell surface recruitment of postsynaptic $GABA_ARs$ in these mature neurons, we next investigated the effects of Akt on the insulin-induced increase in cell surface expression of $GABA_ARs$ using a quantitative cell-ELISA colorimetric assay (Man et al., 2000). As shown in Figure 6G, insulin stimulation (0.5 μM, 5 min) resulted in a significant increase in the proportion of $GABA_ARs$ expressed on the cell surface. This increase is due to a rapid translocation of intracellular receptors to the plasma membrane, as insulin treatment did not alter the total number of $GABA_ARs$ expressed in these cells as measured using either cell-ELISA under cell permeant conditions or as determined by Western blotting of cell lysates with anti-β_2 antibody (data not shown). Consistent with mediation by Akt, the insulin-induced increase in cell surface expression of $GABA_ARs$ was prevented by suppressing Akt activity with LY294002 (10 μM), an inhibitor of Akt-upstream signaling molecule PI3K, but not affected by reducing either PKA or PKC activity (Figure 6G).

The Akt-dependent increase in cell surface $GABA_AR$ expression following insulin treatment might arise from enhanced membrane insertion, or, alternatively, by slowing down the rate of receptor endocytosis. We therefore investigated the contribution of Akt activation to the postsynaptic insertion of $GABA_ARs$ in cultured

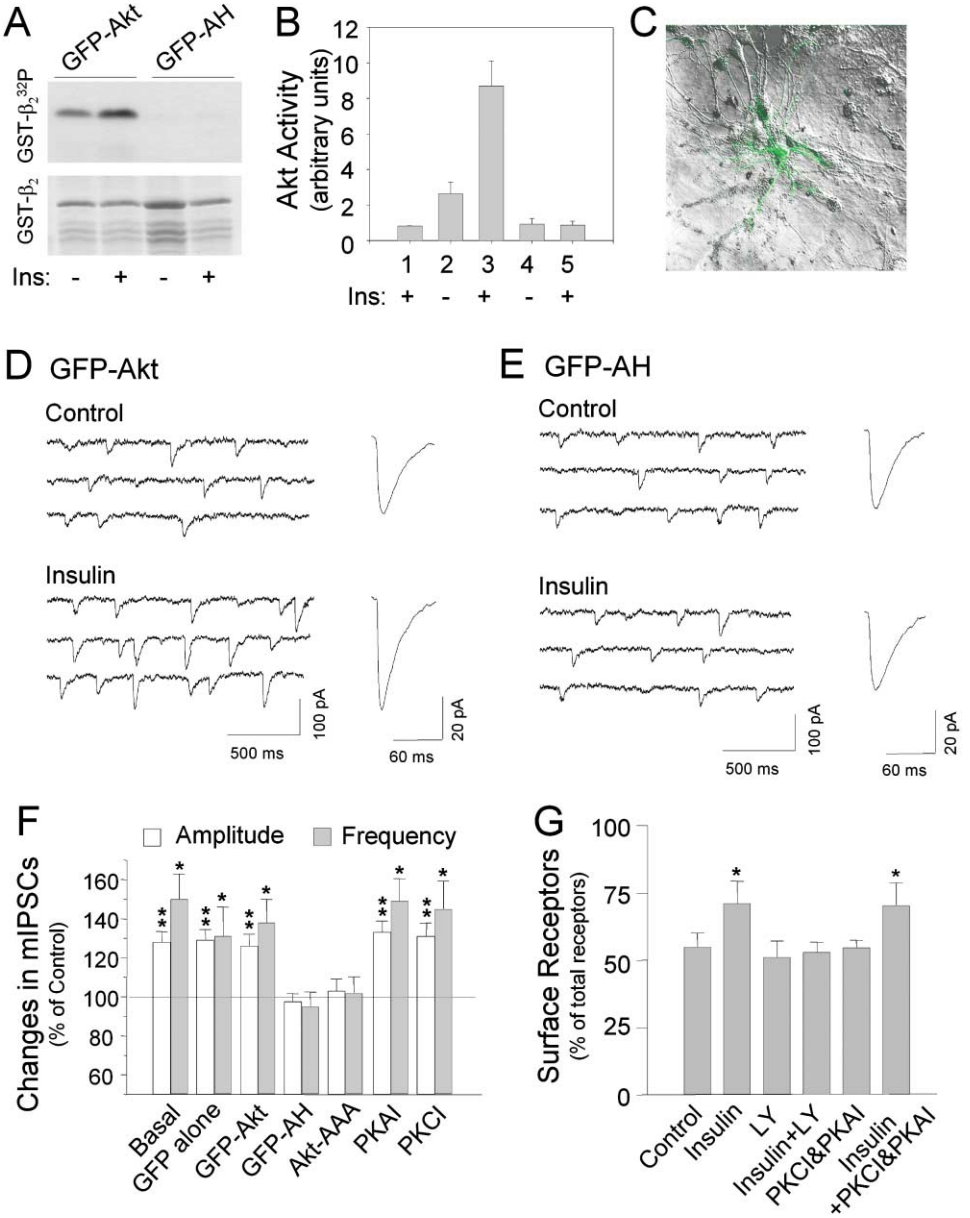

Figure 6. Activation of Akt Potentiates $GABA_A$ Receptor-Mediated mIPSCs in Neurons

(A) GFP-Akt and GFP-AH were immunopurified, using an antibody against GFP, from HEK293 cells transfected with the corresponding constructs and stimulated with (+) or without (−) insulin (Ins; 200 nM, 5 min). Their ability to phosphorylate the GST fusion protein containing the major intracellular loop of β_2 was analyzed using an in vitro phosphorylation assay. Autoradiography of the resultant SDS-PAGE gel and Coomassie blue staining of the same gel are shown on the top and bottom, respectively.

(B) HA-Akt was cotransfected in HEK293 cells with 10-fold higher empty vector (1), GFP-Akt (2, 3), or GFP-AH (4, 5). After the treatment of these cells with (+) or without (−) insulin, HA-Akt was immunopurified using an anti-HA epitope antibody, and kinase activity was determined in vitro using the specific Akt substrate, Crosstide.

(C) Superimposed fluorescent and differential interference contrast images show that a single GFP-AH transfected neuron (green) is surrounded and innervated by a number of nontransfected cells.

(D and E) Whole-cell patch-clamp recordings of $GABA_A$R-mediated mIPSCs from GFP-Akt- (D) or GFP-AH- (E) expressing neurons at a holding potential of −60 mV. Traces on the left are examples of consecutive traces of mIPSCs taken before (Control) and 10 min after insulin treatment (Insulin; 0.5 μM). The middle traces are averages of 200 individual mIPSCs. Akt activation did not alter the decay constants of the averaged mIPSCs: 28.97 ms before versus 29.17 ms after insulin for GFP-Akt-expressing neurons, and 29.55 ms versus 30.19 ms for GFP-AH-expressing neurons.

(F) Histogram summarizing normalized effect of insulin (500 nM, 5 min) on amplitude and frequency of mIPSCs from nontransfected neurons (Control) or neurons transfected with various Akt constructs or neurons incubated with PKA inhibitor (PKAI; Rp-cAMP; 50 μM) or PKC inhibitor (PKCI; Gö6850; 125 nM) 5 min prior to and throughout recordings. Amplitude and frequency of mIPSCs from 3 min continuous recordings following 10 min insulin application (Insulin) were averaged and normalized to (percentage of) their own controls that were obtained immediately before insulin application ($n = 5$ neurons).

(G) Ten minutes after insulin treatment, the neurons were fixed, and $GABA_A$Rs expressed on the cell surface and in the entire cells were then specifically labeled with primary antibody that recognizes the extracellular N-terminal domain of $GABA_A$R α_1 subunit and HRP-conjugated secondary antibodies under nonpermeant and permeant conditions, respectively, and quantified using cell-ELISA assays. The proportion of cell surface receptors was determined using the ratio of absorbance readings obtained under nonpermeant versus permeant conditions ($n = 12$ dishes in each group). * and ** denote $p < 0.05$ and $p < 0.01$, respectively, for the differences between control and treated groups.

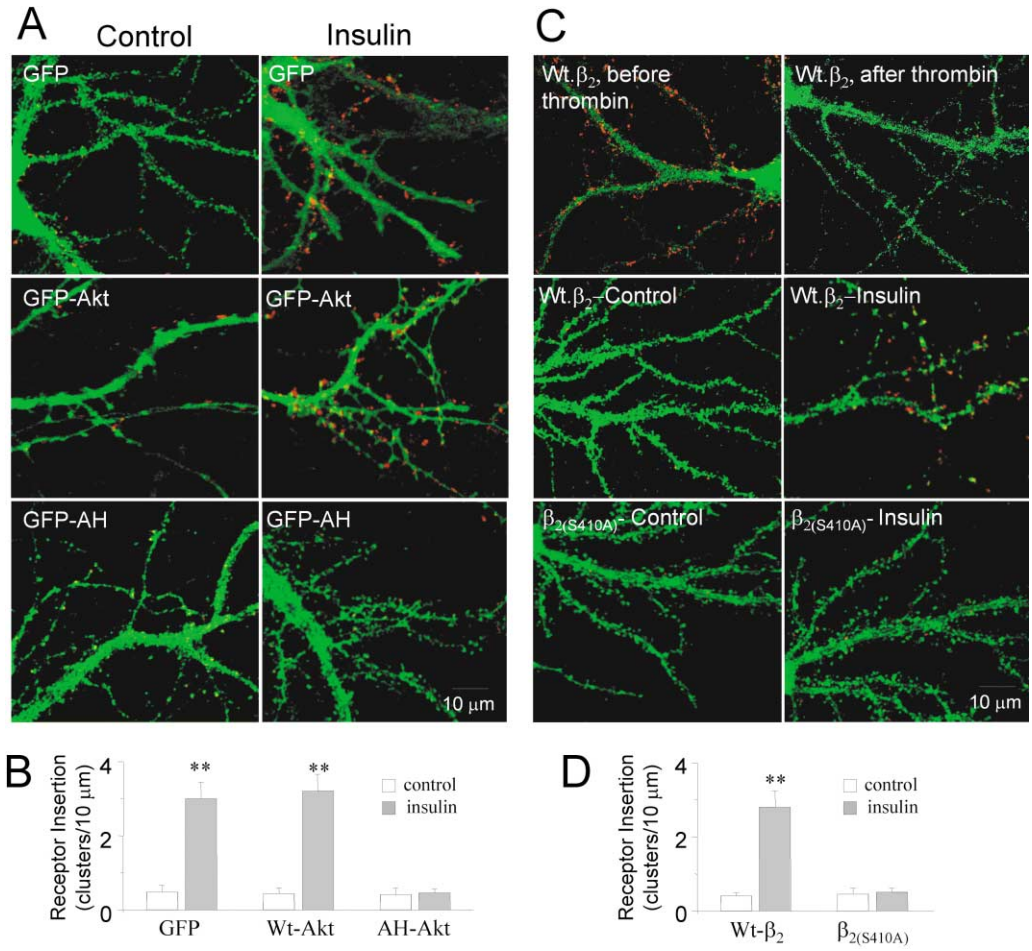

Figure 7. Activation of Akt Increases the Insertion of $GABA_A$ Receptors into Postsynaptic Plasma Membrane in Neurons

(A) GFP, GFP-Akt, or GFP-AH were transfected into cultured hippocampal neurons, and the transfectants were identified by GFP fluorescence (Green). Existing cell surface $GABA_A$Rs were first blocked by an antibody against an extracellular epitope of the β_2 subunit of $GABA_A$Rs and cold (non-fluorescence-tagged) secondary antibody in live cells at 4°C. The insertion of new $GABA_A$Rs was allowed by switching the cells to room temperature in the absence (Control) and the presence of insulin for 10 min (Insulin; 200 nM). Newly inserted receptors were then specifically detected by the same primary anti-β_2 antibody and Cy3-congugated secondary antibody (red) under nonpermeant conditions.

(B) Quantitative analysis of receptor insertion by counting the number of $GABA_A$R clusters (clusters/10 μm).

(C) Mutation of serine 410 on the β_2 subunit prevents Akt activation-induced $GABA_A$R translocation. The $\alpha_1^{Flag\text{-}thr}$, $\beta_2/\beta_{2(S410A)}$, and γ_2, along with GFP as a marker (Green), were cotransfected into cultured hippocampal neurons. Before insulin stimulation, the transfected neurons were treated with thrombin (100 U/ml in ECS), so that the Flag epitope in the α_1 subunit of all receptors at the cell surface under basal conditions was cleaved. After insulin treatment, reinserted Flag-tagged receptor complexes can be detected by a primary anti-Flag antibody and Cy3-conjugated secondary antibodies (Red).

(D) Quantitative analysis of the rate of receptor reinsertion by counting the number of $GABA_A$R clusters (clusters/10 μm). **$p < 0.001$.

neurons expressing either GFP-Akt, GFP-AH, or GFP alone using the method that we have recently developed to specifically visualize newly inserted receptors (Lu et al., 2001). In this assay, native $GABA_A$Rs on the cell surface under basal conditions were first blocked with an antibody against the amino-terminal extracellular epitope of the β_2 subunit and a cold (non-fluorescence-conjugated) secondary antibody at 4°C in live neurons. Following room temperature incubations of varying length, the newly inserted $GABA_A$Rs were then labeled with the same primary antibody and a Cy3-conjugated secondary antibody under nonpermeablizing conditions. As shown in Figure 7A, there were very few clusters of $GABA_A$R staining to be observed in cells examined 10 min after switching the cells to room temperature from 4°C under control, nonstimulated conditions, irrespective of whether the cells were transfected with GFP, GFP-Akt, or GFP-AH. These results not only confirmed the complete blockade of pre-existing $GABA_A$Rs by the cold antibody, but also suggest a relatively slower constitutive (basal) plasma membrane insertion of $GABA_A$Rs, when compared with other ligand-gated neurotransmitter receptors, such as AMPA subtype glutamate receptors (Lu et al., 2001). However, when these cells were switched to room temperature in the presence of insulin (200 nM), there was a time-dependent increase in the number of clusters of $GABA_A$R staining along all the dendrites in every cell expressing either GFP or GFP-Akt (Figure 7A). In sharp contrast, there was no insulin-dependent increase observed in any cells expressing

GFP-AH (Figures 7A and 7B). This observation was repeated in cells transfected with the kinase-dead GFP-ΔAkt construct (data not shown). The results are consistent with a critical role of Akt activation in the insulin-facilitated insertion of native $GABA_A$Rs in neurons in situ.

To determine the role of phosphorylation of β_2 S410 in the Akt-dependent facilitation of $GABA_A$R insertion, we next compared insulin-induced plasma membrane insertion of recombinant $GABA_A$Rs containing the $\beta_{2(S410A)}$ mutant with those containing the wild-type β_2 in transiently transfected hippocampal neurons. Because a recombinant receptor subunit, when transfected into neurons individually, does not heteroligomerize with endogenous receptor subunits, and hence does not traffic to the membrane surface (Gorrie et al., 1997), we cotransfected the wild-type or mutant β_2 subunit with α_1 and γ_2 subunits, along with GFP as a marker for transfectants. In order to facilitate the detection of membrane insertion of the heteropentameric recombinant $GABA_A$Rs with both temporally and spatially high resolutions, we constructed Flag-Thr-α_1 by inserting a Flag-epitope followed by a cleavage site for the protease thrombin at the extracellular N terminus of the α_1 subunit. After transfection into neurons, cell surface expression of Flag-Thr-α_1 containing recombinant $GABA_A$R complexes can be visualized in nonpermeabilized cells by Flag antibodies. In order to selectively detect newly inserted receptors, the Flag signal of the receptors that have already been expressed could be eliminated by treatment of live neurons with thrombin, which cleaves the N terminus, including the Flag-tag. Insertion of new recombinant $GABA_A$Rs from intracellular compartments can then be visualized by detection of the time-dependent reappearance of new Flag-immunoreactivity following thrombin treatment. As shown in Figure 7C, following transfection, nonpermeabilized staining of the Flag epitope showed that the wild-type recombinant $GABA_A$Rs form numerous receptor clusters and accumulated in dendritic and somatic membrane surfaces, with a distribution pattern similar to native $GABA_A$Rs (Figure 4B). Thrombin treatment virtually eliminated cell surface staining of Flag-Thr-$GABA_A$Rs in transfected hippocampal neurons (Figure 7C), confirming the effectiveness of the removal of Flag epitopes from cell surface $GABA_A$Rs. Surface Flag-staining reappeared slowly after thrombin cleavage, and there were only a few clusters accumulated in dendrites within 10 min. Control levels were not reached even 1 hr following the treatment, consistent with a slow rate of constitutive insertion of $GABA_A$Rs demonstrated by the blocking assay shown in Figure 7A. However, insulin stimulation (200 nM) significantly increased the reappearance of cell surface clusters of Flag-staining in neurons transfected with wild-type $GABA_A$Rs (Figures 7C and 7D). Point mutation of β_2 S410 into alanine did not alter the rate of constitutive $GABA_A$R insertion, but it prevented insulin-induced facilitation of receptor insertion (Figures 7C and 7D). The mutation did not appear to affect the level of receptor (protein) expression, as staining of Flag-epitopes under permeabilized conditions showed a level of expression comparable to its wild-type counterparts (data not shown).

Discussion

Our results show that Akt phosphorylates the $GABA_A$R β_2 subunit both in vitro and in vivo, and that this phosphorylation plays an important role in the dynamic regulation of $GABA_A$R numbers at the postsynaptic domain, and hence the efficacy of receptor-mediated synaptic inhibition. As all functional $GABA_A$Rs require a β subunit (Wan et al., 1997a), and the Akt phosphorylation site in β_2 is conserved in all β subunits, Akt-mediated phosphorylation may also be important for insertion of β_1 and β_3 subunit-containing $GABA_A$Rs, representing a common mechanism of regulation associated with all populations of native $GABA_A$Rs in the mammalian brain. Interestingly, β_2 S410 (corresponding to S409 in β_1 and β_3) has also been suggested to be a substrate for other kinases (Brandon et al., 2000; McDonald et al., 1998; McDonald and Moss, 1997; Moss et al., 1992). In an in vitro phosphorylation assay using GST-β_2 intracellular loop as substrate, the β_2 S410 could be phosphorylated by both PKA and PKC (McDonald and Moss, 1997), consistent with its being part of a consensus site for these kinases. However, when β_2 subunit was overexpressed in HEK293 cells, phosphorylation of S410 was only observed after activation of PKC, not PKA, implying that PKC is the primary kinase that phosphorylates β_2 in situ in HEK293 cells (McDonald et al., 1998). This is in contrast to β_1 and β_3, which are phosphorylated by both PKA and PKC in situ at S409 when expressed in the same cell lines (Brandon et al., 2000; McDonald et al., 1998; McDonald and Moss, 1997). These results reinforce the hypothesis that the presence of a consensus site for a particular kinase may not be a sufficient criterion for identifying the protein as a substrate for the kinase in situ. Further to this point, although β_2 S410 has been shown to be phosphorylated in situ following activation of PKC when expressed in HEK293 cells (McDonald et al., 1998), we did not observe any evidence of in situ phosphorylation of the native β_2 subunit at S410 by PKC in cultured hippocampal neurons. Similarly, it has recently been reported that activation of PKA phosphorylates recombinant β_3 subunits in HEK293 cells (McDonald et al., 1998). However, in cultured cortical neurons, the native β_3 subunit is predominantly phosphorylated by PKC, and PKA-mediated phosphorylation was only evident when endogenous PKC activity was inhibited (Brandon et al., 2000). Thus, the same site within the same subunit may be preferentially phosphorylated by different kinases depending on the cell type and the state of the cells. More interestingly, recent studies have reported that, while phosphorylation of β_3 by PKA results in enhancement of $GABA_A$R function (McDonald et al., 1998), phosphorylation of the same subunit by PKC leads to reduced function of the receptor (Brandon et al., 2000). These results suggest that changes in charge density may not be the sole determinant of the functional consequences of phosphorylation and that the particular kinases involved may also play a critical role in dictating the functional consequence of receptor phosphorylation. Thus, it would be interesting to know if phosphorylation of β_2 S410 by PKA or PKC can also enhance plasma membrane insertion of the receptors. Similarly, whether S409 of β_1 or β_3 is also phosphorylated by endogenous Akt in HEK293 cells

and/or neurons, and if so, whether phosphorylation results in increased plasma membrane insertion of the receptors that contain these subunits, remain to be determined.

Causing changes in channel gating such as mean open time and open probability has been thought to be a common mechanism by which receptor phosphorylation alters the function of the receptor, and this has been demonstrated in several ligand-gated channels, including $GABA_A$Rs (Brandon et al., 2000; Hopfield et al., 1988; Moss et al., 1995; Wang et al., 1996). In one of our previous studies using a combination of electrophysiology and electron microscopic immunogold labeling, we were able to demonstrate that insulin produces a rapid translocation of $GABA_A$Rs from the intracellular compartment to postsynaptic domains, thereby potentiating $GABA_A$R-mediated IPSCs (Wan et al., 1997b). In the present study, we extended this work and have shown that this rapid increase in the number of $GABA_A$Rs is a result of Akt-mediated direct phosphorylation of β_2 S410, providing strong evidence for a rapid alteration of the number of $GABA_A$Rs as an important mechanism by which protein phosphorylation alters function of the receptors. Like many integrated plasma membrane proteins, $GABA_A$Rs are thought to be trafficked between the plasma membrane and intracellular compartments via vesicle-mediated plasma membrane insertion and internalization (Moss and Smart, 2001). Thus, increase in the steady-state level of $GABA_A$Rs on the membrane surface could be achieved by either facilitating receptor insertion, inhibiting receptor endocytosis, or increasing receptor stability (intracellular half life and/or plasma membrane anchoring). Indeed, the increase in the number of cell surface $GABA_A$Rs, and hence receptor-mediated current responses, has been demonstrated by inhibitors of clathrin-mediated endocytosis (Kittler et al., 2000) or overexpression of Plic-1, a ubiquitin-like protein, which binds both α and β subunits and enhances the stability of intracellular $GABA_A$Rs, presumably by blocking ubiquitination and subsequent degradation (Bedford et al., 2001). In the present study, using selective measurements of $GABA_A$Rs (both preblocking and thrombin-based cleaving assays), we have been able to demonstrate the insulin-induced increase in plasma membrane insertion of $GABA_A$Rs via an Akt phosphorylation-dependent mechanism. Consistent with this hypothesis, we have recently found that this phenomenon is resistant to inhibition of clathrin-mediated receptor endocytosis (unpublished observation). Thus, the Akt phosphorylation-dependent increase in the number of cell surface $GABA_A$Rs is primarily due to an enhanced receptor insertion. We further demonstrate that phosphorylation of β_2 at the single site, S410, is a prerequisite for increased $GABA_A$R insertion, and hence potentiated mIPSCs. Our work has therefore provided strong evidence suggesting that direct receptor phosphorylation by Akt may be an important mechanism underlying the dynamic regulation of $GABA_A$R numbers at postsynaptic sites, and hence synaptic strength at GABAergic synapses.

However, the physiological implications of the present work clearly extend beyond modulation of $GABA_A$R-mediated synaptic transmission. Akt is a common component in signaling pathways used by many extracellular molecules, including growth factors, cytokines, and neurotransmitter receptors (Dudek et al., 1997; Morisco et al., 2000; Yano et al., 1998), as well as intracellular factors such as Ras (Kauffmann-Zeh et al., 1997) and Ca^{2+} (Yano et al., 1998). Akt phosphorylation sites are also present in other ionotropic neurotransmitter receptors. Modulation of intracellular trafficking and function of ionotropic neurotransmitter receptors by Akt may be a common mechanism by which diverse extracellular and intracellular signaling pathways modulate neuronal functioning in the brain. In this regard, it is interesting to note that serine phosphorylation of GluR1, a subunit of AMPA subtype glutamate receptors, has been shown to be critically important for activity-dependent insertion of postsynaptic AMPA receptors during the expression of the homosynaptic hippocampal CA1 long-term potentiation (LTP), a well-characterized synaptic plasticity that has been proposed as a cellular substrate for learning and memory (Malenka and Nicoll, 1999; Malinow et al., 2000). Although the underlying serine kinase has not been clearly identified, several recent studies have provided strong evidence suggesting an absolute requirement of PI3K, a critical kinase upstream of Akt activation (Downward, 1998), in LTP (Kelly and Lynch, 2000; Man et al., 2003; Sanna et al., 2002). These studies warrant further examination to determine whether Akt can function as one of the serine/threonine kinases that phosphorylates GluR1, and hence mediates AMPA receptor insertion during LTP expression. In addition, Blair and Marshall have recently reported that insulin-like growth factor-1 potentiates the N and L type voltage-gated calcium channels via an Akt-dependent mechanism (Blair et al., 1999; Blair and Marshall, 1997), and Lhuillier and Dryer (2002) have also demonstrated an involvement of Akt in TGF β_1 regulation of a calcium-activated potassium channel. But, in both cases, the detailed mechanisms by which Akt exerts its actions have not been established, and whether these channels are direct substrates for the kinase or indirect targets for Akt-dependent signaling pathways remains to be defined. By identifying the $GABA_A$R as a direct substrate of Akt, the present work provides strong evidence suggesting that ion channels may represent a novel class of substrates for Akt, and that by direct phosphorylation, Akt can regulate function of these ion channels. Thus, the present study not only links the ubiquitous serine/threonine kinase to synaptic plasticity, but also provides a mechanistic framework for studying the detailed mechanisms underlying Akt modulation of other ligand-gated, as well as voltage-gated, ion channels. Both ligand-gated and voltage-gated ion channels have a fundamental role in regulating neuronal functioning through the control of neuronal excitability. Through phosphorylation and modulation of these channels, Akt, in addition to its known function in supporting neuronal survival, may have profound actions on neuronal excitability, thereby playing important roles in physiological and pathophysiological processes in the mammalian brain.

Experimental Procedures

Akt Kinase Assays

Mammalian expression vectors encoding wild-type Akt (pSG5-HA-PKB), kinase-dead ΔAkt (pSG5-HA-PKB^{K179M}), GFP-Akt, and GFP-

AH were generously provided by Dr. Julian Downward at the Imperial Cancer Research Fund, London, UK. The mammalian vector encoding the conditionally active Akt (ER-Akt) was a gift from Dr. R. Roth at Stanford University, Stanford, CA. HEK293 cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum. Transfections were performed in 10 cm dishes using the Effectene transfection kit according to the protocol suggested by the manufacturer (Qiagen, Mississauga, ON). Forty-eight hours after transfection, cells were serum deprived for 4 hr and treated with or without insulin (200 nM; 5 min) or hydroxyltamoxifen (HT; 1 μM, 30 min). Treated or untreated cells were harvested in a lysis buffer. The various forms of Akt were immunoprecipitated with an anti-HA antibody (Covance; 3 μg), and their kinase activity was then determined in an immunocomplex assay using Crosstide as a specific Akt substrate as described (Wang et al., 1999).

In Vitro Phosphorylation of GST Fusion Proteins

Construction of pGEX2T vectors encoding GST-fusion proteins of the intracellular loop between transmembrane domains 3 and 4 of rat $GABA_AR$ α_1 (GST-α_1), β_2 (GST-β_2), or γ_2 (GST-γ_2) subunits have been described previously (Liu et al., 2000). GST-$\beta_{2(S410A)}$ and GST-$\beta_{2(3Y\text{-}A)}$ (Y304A, Y372A, Y379A) constructs were generated by mutating the corresponding residue(s) of the GST-β_2 construct into alanine(s) using a QuickChange™ Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA), and all mutations were confirmed by sequencing. In vitro phosphorylation of GST fusion proteins was performed essentially as described for in vitro Akt kinase assays in the text and the legend for Figure 1 except that GST fusion proteins (1 μg for each reaction) were used as substrates instead of Crosstide.

In Vivo Phosphorylation in HEK293 Cells

Mammalian expression vectors encoding wild-type rat $GABA_AR$ subunits β_2, γ_2 (short splice variant), and the N-terminal extracellularly Flag-tagged rat α_1 have been previously described (Wan et al., 1997b). To generate the mammalian expression vector pcDNAI-β_2^{myc} that encodes extracellularly myc-epitope-tagged rat β_2 subunit, the myc epitope (EQKLISEEDL) was inserted by PCR into the pcDNAI-β_2 vector immediately before the stop codon of the β_2 coding sequence. The serine-alanine conversion was then introduced into the plasmid to generate the pcDNAI-$\beta_{2(S410A)}^{myc}$ construct using the QuickChange™ Site-directed Mutagenesis Kit (Stratagene). Transfection of HEK293 cells with various plasmid combinations was done using the Effectene transfection kit (Qiagen). Following serum starvation, cells were labeled with [^{32}P]orthophosphate (100 μCi/ml, NEN) for at least 3 hr in phosphate-free culture medium, and then subjected to various drug treatments as described in the main text and the legend of Figure 2. Heteromeric $GABA_ARs$ were immunoprecipitated, using an anti-Flag antibody, from lysates of cells expressing α_1^{Flag}, β_2, and γ_2 subunits under nondenaturing conditions in Buffer A (50 mM HEPES [pH 7.6], 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride [PMSF], aprotinin, leupeptin). The β_2 subunit was isolated by immunoprecipitating the lysates of cells expressing α_1, β_2^{myc}, and γ_2 with anti-myc antibody under denaturing conditions (Buffer A plus 1 mM DTT and 0.1% SDS). The immunoprecipitates were resolved by 10% SDS-PAGE, and incorporation of ^{32}P into the corresponding protein bands was detected by autoradiography.

In Vivo Phosphorylation in Hippocampal Neuronal Cultures

Cultured hippocampal neurons were prepared and maintained as described previously (Man et al., 2000). Hippocampal cells ($\sim 2 \times 10^7$) were homogenized in buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% igepalCA630, 0.5% sodium deoxycholate, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, and protease inhibitor cocktail (Sigma 5 μl/100 mg tissue) and centrifuged at 10,000 × g at 4°C for 20 min. Membrane pellets were further solubilized for 1 hr on ice with 1% Triton X-100 and centrifuged at 48,000 × g for 20 min. The supernatant was extracted and protein concentrations were measured. The lysates (500 μg protein) were then immunoprecipitated using a monoclonal anti-$GABA_AR$ β-chain antibody (~1–2 μg, Chemicon, Temecula, CA) for 4 hr at 4°C, followed by addition of 20 μl of protein A/G Sepharose (Amersham Biosciences, Baie D'Urfé, PQ) for 12 hr. The precipitates were then subjected to SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with anti-phospho-(Ser/Thr) Akt substrate (PAS) antibody (1:1000, New England Biolabs, Mississauga, ON) overnight at 4°C. HRP-conjugated anti-rabbit IgG was used as a secondary antibody and bound antibodies were detected by ECL (Amersham).

Confocal Imaging and Cell-ELISA Measurement of the Subcellular Distribution of $GABA_ARs$

Cells were fixed with 2% paraformaldehyde in PBS for 10 min, 48 hr posttransfection. Surface $GABA_ARs$ were labeled with a monoclonal anti-Flag antibody (1:500; Sigma, St. Louis, MO) and visualized with a Cy3-conjugated anti-mouse IgG antibody (1:600; Jackson Labs). Intracellular $GABA_ARs$ were stained with an anti-Flag antibody and visualized with FITC-labeled anti-mouse antibodies following permeabilization of the cells with 0.25% Triton X-100 in PBS for 10 min. Quantification of cell surface $GABA_ARs$ was performed by a colorimetric cell-ELISA assay described previously (Man et al., 2000). Briefly, for transiently expressed recombinant $GABA_ARs$ in HEK293 cells, the cells were transiently transfected with α_1^{Flag}, β_2, or γ_2 with or without various Akt cDNAs in 10 cm dishes and were reseeded into 12-well plates at a seeding density of 10^6 cells/well 24 hr posttransfection. Cells were used 48 hr after transfection. Following a 1–3 hr period of serum starvation, HEK293 cells or cultured hippocampal neurons were treated as described in the text and corresponding figure legends and fixed (3 min in PBS containing 2% paraformaldehyde for nonpermeant conditions, 15 min in PBS containing 4% paraformaldehyde for permeant conditions). $GABA_ARs$ on the plasma membrane surface and total cellular receptor levels were then determined by incubating the cells under nonpermeant and permeant conditions, respectively, with anti-Flag antibody (1:500) for recombinant $GABA_ARs$ in HEK293 cells, or a polyclonal antibody against the extracellular N terminus of rabbit $GABA_AR$ α_1 subunit (1 μg/ml; Chemicon) for native $GABA_ARs$ in cultured neurons, overnight at 4°C. The cells were then incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibody (1:1000, Amersham Biosciences) for 1 hr at room temperature. Following extensive washing in PBS, cells were incubated with OPD substrate (Sigma) for approximately 10 min. Reactions were stopped using 0.2 volumes of 3 N HCl, and 1 ml of supernatant was used to determine absorbance at 492 nm.

Plasmid Transfection and Electrophysiology in Primary Neuron Cultures

Neurons were transiently transfected with GFP-Akt or GFP-AH 14 days after plating using the Effectene transfection kit (Qiagen) according to the protocol suggested by the manufacturer. Transfected neurons were identified using epifluorescent microscopy (Wan et al., 1997a). Only GFP-positive transfected neurons that could be clearly identified and were innervated by nontransfected neurons were used for whole-cell voltage-patch clamp recordings. $GABA_AR$-mediated mIPSCs were recorded and analyzed as described previously (Wan et al., 1997b).

Thrombin Cleavage of FLAG-Tagged Expressing Vectors

Primary cultures of hippocampal neurons were transfected with cDNAs for the $\alpha_1^{Thr\text{-}Flag}$ (encoding an extracellular N-terminal Thrombin-FLAG), β_2, and γ_2 subunits of the $GABA_AR$ and various Akt plasmids using Effectene (Qiagen). Transfected neurons were placed in extracellular solution (ECS) and incubated at 10°C for 30 min in order to reduce receptor trafficking. The N-terminal region of the α_1 subunits was cleaved by incubation with thrombin (100 U/ml in ECS) for 30 min at 10°C. Cells were allowed to recover in ECS at room temperature for 15 min before insulin stimulation. Following fixation (4% paraformaldehyde in PBS), cells were extensively washed with glycine (0.1 M in PBS) and blocked with 1% normal goat serum (1 hr, room temperature). The reinserted receptors were labeled with primary anti-FLAG antibody (1:500, overnight at 4°C) and visualized with secondary Cy3-conjugated anti-mouse antibodies (1:1000, 30 min) under laser scanning microscopy.

Preblocking of Surface $GABA_ARs$ Containing Endogenous α_1 Subunits

Primary hippocampal cultures were placed in ECS and cooled to 4°C for 30 min. Neurons were incubated with rabbit IgG (1:100;

Upstate Cell Signaling Solutions, Lake Placid, NY) against the extracellular N-terminal region of the α_1 subunit for 30 min at 4°C. Unbound primary antibody was removed by washing and bound antibody was neutralized by incubation with ice-cold ECS containing unlabeled secondary anti-rabbit antibody (1:100 in ECS) for 30 min. Cells were then rinsed briefly with ECS and incubated with or without insulin for 10 min. After an additional 15 min incubation in ECS at 37°C, neurons were fixed in 4% paraformaldehyde and then blocked with 1% normal goat serum in PBS for 1 hr at room temperature. Newly inserted receptors were labeled with the anti-α_1 $GABA_AR$ IgG (1:300) overnight at 4°C. Cells were then washed with PBS and incubated with secondary Cy3-conjugated anti-rabbit antibodies for 30 min at room temperature. Following washing, the cells were mounted and viewed by laser scanning microscopy.

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